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Award Number: W81XWH-07-1-0055

TITLE: Role of Katanin in Prostate Cancer Bone Metastasis

PRINCIPAL INVESTIGATOR: Xiang-Cang Ye, Ph.D.

CONTRACTING ORGANIZATION: M. D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: January 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
17-JAN-2008	Annual	18 DEC 2006 - 17 DEC 2007

4. TITLE AND SUBTITLE
Role of Katanin in Prostate Cancer Bone Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0055

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Xiang-Cang Ye, Ph.D.

Email: xcye@mdanderson.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

M.D. Anderson Cancer Center
Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We previously identified katanin p60 as a differentially expressed protein in the bone marrow samples from prostate cancer patients with clinical evidence of bone metastasis. In order to explore the functions of katanin p60 in prostate cancer, we carried out molecular cloning and characterization of katanin p60. From prostate cancer tissues, we cloned three alternative splicing forms in addition to the full-length katanin p60. Two of isoforms showed an effect in modulating cell migration/proliferation in a wound-healing test. Meanwhile, we established stable cell lines and *in vitro* study systems for future studies. We will continue to characterize the functionalities of katanin p60 and isoforms by use of shRNA to down-regulate the endogenous katanin p60 in prostate cancer cells, so that to compare with those contrastingly over-expressing katanin p60 or isoform. Moreover, as planed in Statement of Work, we will conduct a correlative study of the cellular and tissue distribution of katanin p60 and isoforms in different stages of prostate cancer. Thus, the final outcome of this study will help us to understand the mechanism of katanin-mediated cell activity and to find relevant targets for cancer therapy.

15. SUBJECT TERMS
prostate cancer, metastasis, katanin, cell motility

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

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Role of Katanin in Prostate Cancer Bone Metastasis

Introduction

Prostate cancer is the second most common cause of cancer-related death among men in the United States. The late stage of androgen-refractory prostate cancer is dominated by complications arising from bone metastasis. To date, there is no effective treatment for bone metastases.

Our preliminary study using the proteomics approach has identified katanin p60 as a differentially expressed factor in the bone marrow samples from prostate cancer patients with clinical evidence of bone metastasis. According on the published literatures, katanin p60 is a member of AAA (ATPases associated with various cellular activities) protein family. It has been known as a subunit of katanin heterodimer having a microtubule-severing activity in the centrosome. It has biological functions involving in mitotic cell division and neuronal migration. However, its relationship with the cancer bone metastasis has never been reported.

We hypothesize that katanin p60 serves as a cell migration factor to mediate prostate cancer metastasis to bone. We will focus on the characterization of the katanin p60 *in vitro* and *in vivo* with regards to its functions in prostate cancer bone metastasis.

Body

Our research has been carried out according to the approved Statement of Work. In this period of time (Months 1-12, as outlined below), our task is to accomplish the gene cloning, stable cell line generation and primary *in vitro* functional study.

Task 1: To determine the function of katanin p60 in cell motility. (Months 1-15)

- Cloning of the alternative spliced katanin p60 cDNA from PCa cells if confirmed. (months 1-2)
- Generation of cDNA expression constructs and recombinant retrovirus. (months 3-6)
- Generation of stable cell lines for overexpression of katanin p60. (months 7-9)
- Characterization of stable cell lines overexpressing katanin p60 *in vitro*. (months 10-12)
- Assessment of the effects of katanin p60 on cell motility by RNA intereference. (months 13-15)

We started the project by identification of the alternative splicing forms of katanin p60. As rationale explained in our proposal, there is possibility that an alternative splicing form of katanin p60 may be involved in modulation of microtubule organization and distinguish its function in cell migration from that in cell division. To identify katanin p60 gene's alternative splicing transcripts, we established a reverse transcription - polymerase chain reaction (RT-PCR) for amplification of specific complementary DNA (cDNA) derived from the samples of PC-3 xenograft tumors and clinical prostate cancer tissues (PCa-S1 and PCa-S2). The RT-PCR was carried out with *Taq* polymerase and the primers embracing a region from exon 4 to exon 8 (Figure 1). Following the subcloning and DNA sequencing of the katanin p60 amplicons from these samples, we found at least two alternatively spliced transcripts in addition to KATNA1, which encodes the full-length katanin p60 (GenBank accession NM_007044). These two alternative splicing forms contain the exon 7 deletion (KATNA1-ΔE7) or the exon 5 to exon 7 deletion (KATNA1-ΔE5-7), respectively.

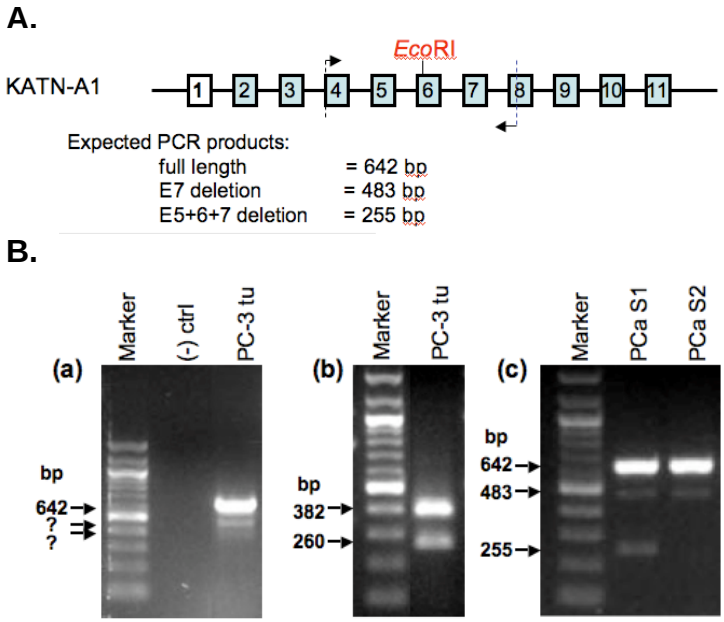

Figure 1. RT-PCR of katanin p60. ***A.*** Primers specific to sequences at the exon boundary 4-5 and at the exon 8 were used in PCR. ***B.*** **(a)** RT-PCR of PC-3 xenograft tumor (PC-3tu). Note the smaller amplicons. **(b)** Restricition digestion of PC-3tu amplicon by *Eco*RI. **(c)** RT-PCR of two individual prostate cancer cDNA samples (Origene). The amplicons were cloned and sequenced.

To order to isolate the full-length cDNA clones of katanin p60 isoforms from prostate cancer sample, we designed several pairs of PCR primers specific to the noncoding sequences at exon 1 and exon 11 (Figure 2). RT-PCR of prostate cancer cDNA samples was performed with high fidelity DNA polymerase *pfu* Ultra^{TM} Hotstart (Stratagene). The PCR products were cloned into pcDNA3.1D/V5-His vector (Invitrogen) according to manufacture instruction. Individual clones were screened by restriction digestion and then were confirmed by DNA sequencing. From three batches of PCR cloning, we obtained the full-length cDNA of KATNA1 and three alternative splicing isoforms, which contain the exon 7 deletion (KATNA1-ΔE7), the exon 10 deletion (KATNA1-ΔE10), or the exon 7 and 10 dual deletion (KATNA1-ΔE7,10) (Figure 2).

However, we did not found KATNA1-ΔE5-7 or the previously known isoform KATNA1-ΔE5,6,10 (GenBank accession BC050428) in our PCR cloning pools. Possibly, they may use an alternative upstream or downstream exon that mismatches to our PCR cloning primers. So far, we have failed in all attempt so far to find such an alternative exon. Another possibility is that the PCR cloning with *pfu* DNA polymerase might be insensitive to those very minor species among the total RNA.

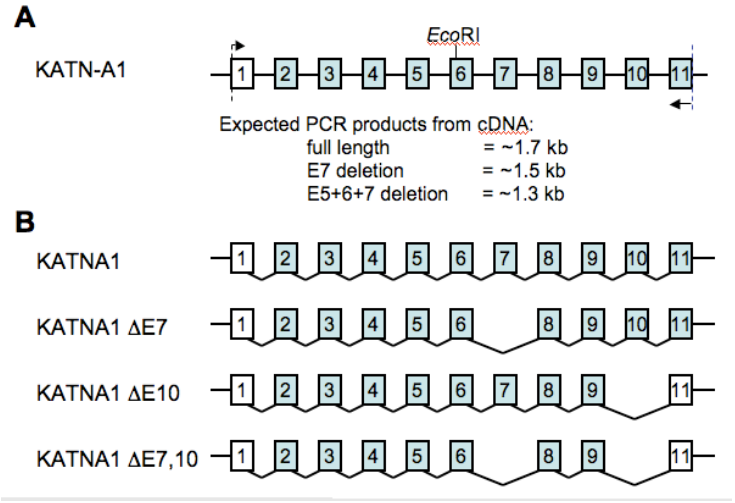

Figure 2. Isolation of katanin p60 and its alternative splicing isoforms from prostate cancer samples. ***A.*** RT-PCR cloning strategy. ***B.*** The cDNA clones of katanin p60 and its isoforms. Exon 1 has no coding sequence. Exon 11 has early termination in the transcripts containing ΔE10 due to open-reading frame shift.

While continuing to search for other katanin p60 isoforms, we have generated cDNA constructs of KATNA1, KATNA1-ΔE7, KATNA1-ΔE10, and KATNA1-ΔE7,10. These constructs were initially made in expression vector pcDNA3.1D/V5-His and subsequently subcloned into recombinant retroviral vector pBMN-IRES-Tomato (Figure 3). pBMN-IRES-Tomato is a bicistronic viral vector for expressing a gene of interest in tandem with a reporter gene via an internal ribosome entry site (IRES). This vector was modified from pBMN-IRES-GFP (a kind gift from Gary Nolan, Stanford University) by replacement of the GFP gene with a gene encoded for Tomato red fluorescent protein (provided by Roger Y. Tsien, University of California at San Diego). With this vector, co-expression of katanin p60/isoform and Tomato Red can be monitored by fluorescent microscope.

The viral preparation procedure has been successfully established in our laboratory recently. In order to generate recombinant retrovirus, we started with transfection of the pBMN-based plasmids into Phoenix Ampho cells (ATCC product# SD 3443) by using Fugene 6 (Roche Diagnostics). The virus-containing supernatant was collected 48 hrs later and concentrated 10-fold by using a Centriplus concentrator (Millipore).

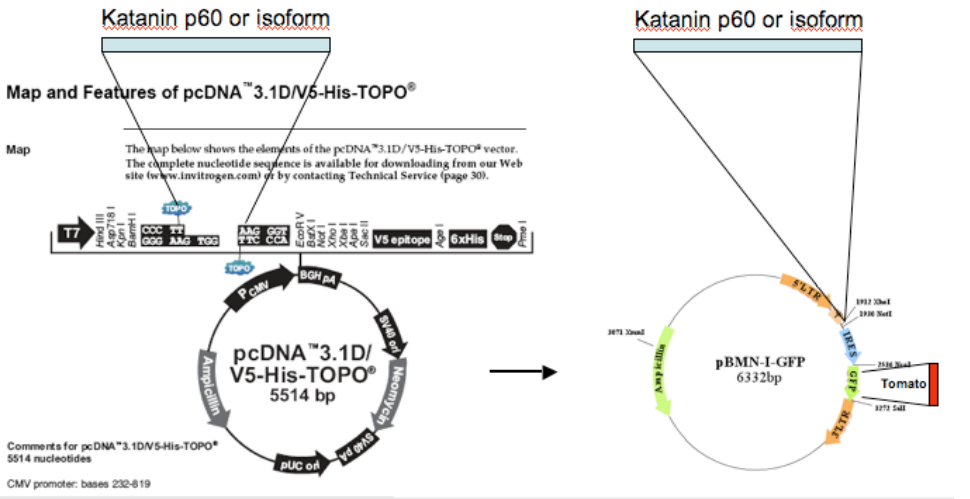

Figure 3. Diagram of a pcDNA3.1-based plasmid and a pBMN-based viral construct for expression of katanin p60 and its isoforms.

We used the recombinant virus for generation of stable cell lines that overexpress katanin p60 or its isoform. The procedure was carried out by transduction of prostate cancer cells with the recombinant retrovirus in the presence of polybrene at 32°C for 24 h. The cells were selected by fluorescence-activated cell sorting on a FACScan (Becton Dickinson). We obtained high homogeneity of transduced stable cell lines that were consisting of more than 90% cells positively expressing reporter. Two of the transduced and sorted cell lines, C4-2b/i-Tom (vector control) and C4-2b/Katn-i-Tom (overexpression of full-length KATNA1), are shown in Figure 4.

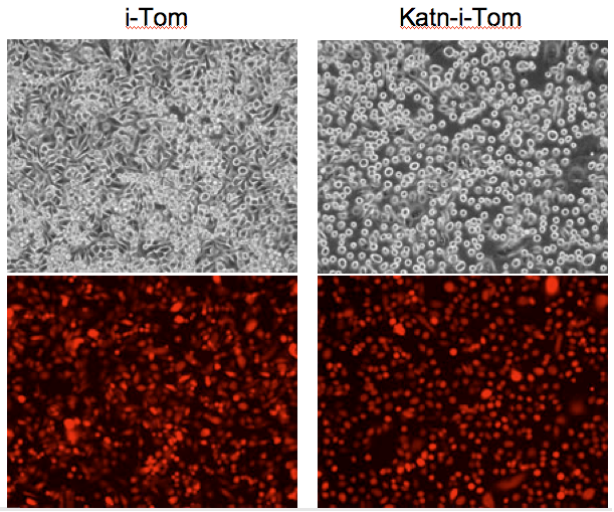

Figure 4. Transduced C4-2b prostate cancer cell lines. Top two panels show light-field images of the cell lines; Note the i-Tom cells were transduced by the virus carrying only a Tomato reporter; whereas the Katn-i-Tom cells were transduced by the virus having both KATNA1 and the Tomato reporter. Bottom panels showed the fluorescent images of two corresponding cell lines.

While we were in process for generating recombinant virus and making stable cell lines, we tested the transient overexpression of katanin p60 and isoforms in prostate cell lines. In transient transfection assays, we used the early generated pcDNA3.1D/V5-His-based plasmids, in which the V5-His epitope is tagged at the C-terminus of a cloned katanin p60 gene product. To achieve high transfection rate in prostate cells, we used electroporator (Amaxa) to deliver plasmid DNA. The control cells were transfected with GFP plasmid and usually produced 80~90% green fluorescent positivity in every batch of experiment. Expression of the full-length KATNA1 in the transfected cells was confirmed by Western blotting with V5-specific antibody (Figure 5). However, the alternative splicing forms contain ΔE10 could not be detected by the V5-immunoblotting because the exon 10 deletion causes open-reading frame shift and results in early termination in C-terminus.

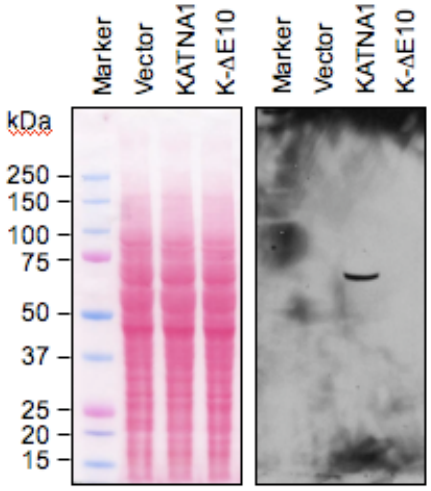

Figure 5. Western blotting of cell lysates. BPH1 cells were transfected with plasmid pcDNA3.1D/V5-His (as vector control) or pcDNA3.1D-KATNA1 or pcDNA3.1D-KATNA1-ΔE10. The cell lysates were used in gel electrophoresis. Comparable total protein loadings were visualized by Ponseus S staining on the transferred membrane (shown at right panel). The V5-tagged katanin p60 was positively detected by V5 immuno-blotting (left panel).

We used these transient transfected cells in the wound-healing test, which could give us an indication whether overexpression of katanin p60 or its isoform affects on cell motility. One of experiments was done in a pre-malignant prostate cell line, BPH1. Result showed that transient overexpression of full-length KATNA1 rendered relatively faster fill-up on the wound line; whereas isoforms KATNA1-ΔE7 and KATNA1-ΔE10 showed

slower fill-up (Figure 6). The dual deletion isoform KATNA1-ΔE7,10 had no effect. However, it has yet been confirmed that whether the KATNA1-ΔE7,10 could produce a stable protein. This preliminary study provided us a first clue of potential functions of katanin p60 and its isoforms in regulation of cell motility.

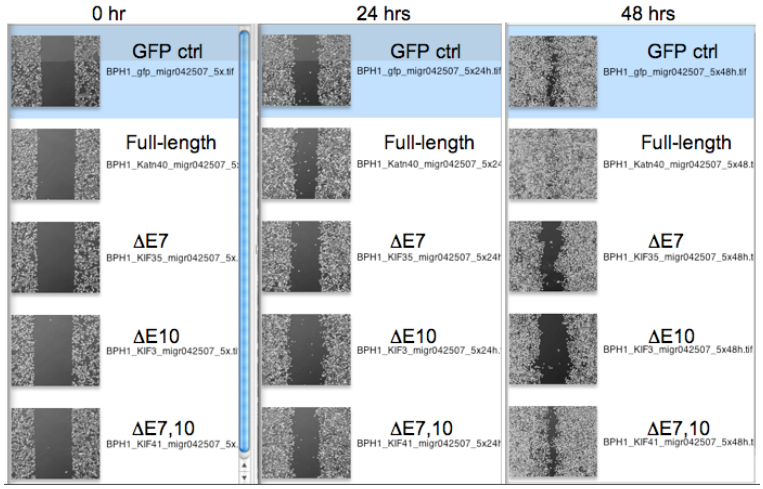

Figure 6. Wound-healing test with the BPH1 cells that were over-expressing katanin p60 or its isoform. Transient over-expression of respective genes was achieved by electroporation method. Serial images were taken at the time as indicated on each column.

Key Research Accomplishments

- Identified four alternative splicing forms of katanin p60 transcripts (KATNA1-ΔE7, KATNA1-ΔE5-7, KATNA1-ΔE10, KATNA1-ΔE7,10) from human prostate cancer samples.
- Cloned the full-length katanin p60 (KATNA1) cDNA and three alternative splicing forms (KATNA1- ΔE7, KATNA1-ΔE10, KATNA1-ΔE7,10) cDNAs from human prostate cancer samples.
- Created pcDNA3.1D-based expression plasmids and pBMN-based retroviral constructs for KATNA1/isoform.
- Generated recombinant retrovirus carrying KATNA1/isoform and successfully used in prostate cancer cell transduction.
- Generated a series of stable cell lines from C4-2b cell background by retroviral transduction of KATNA1/isoform.

- Characterized overexpression of KATNA1/isoform from transient transfection.
- Tested cellular response to overexpression of KATNA1/isoform in transient transfected cells.

Reportable Outcome

Several gene clones, expression plasmids, viral reagents, stable cell lines have been produced during this period of time. However, at this stage of study, the properties of these products need to be further characterized. Documentation and manuscript are in preparation.

Conclusion

We have identified four alternative splicing forms of katanin p60 in prostate cancer samples. Based on structural information from references, these isoforms are apparently intact at N-terminal part but have alterations in some middle segments or at C-terminus. We predicate these alterations may not affect the proteins interaction with the N-terminal binding partner, katanin p80, which serves as a scaffold subunit to bring katanin p60 to centrosome. However, these alterations likely render some changes in their ATPase activity and subsequently affect on the microtubule-severing activity. In our wound-healing test, the full-length KATNA1 enhanced cell migration/proliferation; while its isoforms KATNA1-ΔE7 and KATNA1-ΔE10 showed an inhibitory effect, suggesting these two isoforms may play a role in modulating katanin activity and thus the microtubule cytoskeleton reorganization.

We have established essential *in vitro* systems for this project. Further studies are needed to characterize the protein-protein interactions and the functionality of katanin p60 isoforms. Different cell migration and proliferation assays will be carried out with the stable cell lines. Also, we will use shRNA to knock down the endogenous katanin p60 in prostate cancer cell lines to compare with those contrastingly over-expressing katanin p60 or isoform. Moreover, as planed in Statement of Work, we will conduct a correlative study of the cellular and tissue distribution of katanin p60 and isoforms in different stages

of prostate cancer. Thus, the final outcome of this study will help us to understand the mechanism of katanin-mediated cell activity and to find relevant targets for cancer therapy.

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Appendices

None.